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5P3

GMP reductase modulates purine nucleotide concentrations and uncoupling protein 1 (UCP1) activity

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In brown adipose tissue (BAT), uncoupling protein 1 (UCP1) is the central component of non-shivering thermogenesis that provides heat to defend body temperature in the face of low ambient temperatures. UCP1 facilitates a proton flux across the mitochondrial inner membrane thereby uncoupling respiration from ATP synthesis. In the resting state purine di- and triphosphate nucleotides (GDP, GTP, ADP, ATP) repress UCP1 activity, while fatty acids liberated upon adrenergic activation overcome this inhibition and activate UCP1 upon cold exposure. So far, cold induced alterations in cellular nucleotide concentration are not thought to play a role in UCP1 regulation. In BAT, guanosine monophosphate reductase (GMPR) is strongly upregulated upon cold exposure on both the mRNA and the protein level. This enzyme catalyzes the reaction of GMP back to the common presurcor of both guanosine and adenosine nucleotides, inosine monophosphate (IMP). We aimed to unravel a possible role of GMPR and an altered purine nucleotide metabolism in the regulation of UCP1 activity.

Concordant with its molecular function, forced expression of GMPR in a heterologous expression system led to a shift in the ratio between adenosine and guanosine nucleotides without a disturbance in overall cellular energy balance. In the presence of UCP1, however, the GMPR mediated A/G shift was accompanied by a loss in triphosphate nucleotides indicating an impaired energy supply. Furthermore, in the presence of both UCP1 and GMPR cells displayed an increased basal and fatty acid induced proton leak respiration. Both findings imply that the enzymatic action of GMPR leads to an increased UCP1-mediated proton leak by altering purine nucleotide concentrations.

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5P4

Evidence for a brown adipocyte specific enhancer in the first intron of the murine Ucp3 gene

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Uncoupling protein 3 (UCP3) is a mitochondrial carrier with multiple assigned functions including the transport of protons to reduce ROS formation and modulate calcium homeostasis, the export of excess free fatty acids or lipid radicals and the transport of chloride or pyruvate. UCP3 is expressed but differentially regulated in skeletal muscle (SKM) and brown adipose tissue (BAT). Initial characterization of the basal promoter revealed binding sites for PPARγ, MyoD and thyroid receptor but the underlying mechanisms differentiating gene expression between BAT and SKM are not known to date. Our group identified an intronic element required for Ucp3 expression in BAT. Subsequently a PPAR response element (PPRE) in juxtaposition of our intronic element was found.

Using band shift assays, RNA interference and reporter gene assays we demonstrated SP1/SP3 binding to our intronic element in two brown adipocyte cell lines. Inhibition of SP factor DNA binding either by site directed mutagenesis or mithramycine treatment abolished PPAR agonist activation of reporter gene constructs. Selective deletion of the known PPREs revealed that the intronic PPRE next to our SP binding site is sufficient and required for PPAR agonist activation. Transactivation was solely conveyed by the PPARγ agonist rosiglitazone while neither PPARα nor PPARδ agonists were of relevance in our system. Finally stepwise deletions covering the whole first intron assisted by bioinformatics revealed a putative MyoD binding site right next to the dual SP/PPAR element.

Using the very same reporter constructs, PPAR stimulation, regardless of PPAR subtype, was not able to activate our reporter gene constructs in C2C12 cells. This is well in line with data from SKM or C2C12 cells where mutation of the SP1/3 element had no effect on UCP3 expression. As published data demonstrate that endogenous UCP3 mRNA expression can be induced by PPARγ agonists in those cells, we conclude that PPAR action in SKM, in contrast to BAT, is neither mediated by the intronic PPRE nor by the PPRE in the core promoter.

Taken together, we propose a complex enhancer region located within the first intron. This module, consisting of at least SP, PPAR and MyoD binding sites, does not only confer the responsiveness of UCP3 to PPARγ stimulation in BAT but also differentiates regulation of expression between skeletal muscle and brown adipose tissue.

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5P5

Phospholipase iPLA$_2$γ–dependent regulation of uncoupling protein UPC2 in insulinoma INS-1E cells

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We tested a hypothesis that reactive oxygen species (ROS)-dependent activation of mitochondrial phospholipases leads to an increase in respiration and to more intensive attenuation of mitochondrial ROS production due to UCP2-dependent uncoupling. This was tested on the model of pancreatic β-cells, INS-1E cells, by using the loss-of-function approach through UCP2 silencing. Oxygen consumption was assayed in intact INS-1E cells under varying levels of glucose after overnight incubation in low glucose (3 mM). Glucose addition exhibited a steady increase in respiration which was further stimulated by sub-millimolar concentrations of tert-butyl hydroperoxide (TBHP, 0.25 – 1 mM). The effect of TBHP was significantly lower in UCP2-silenced cells than in control cells. The TBHP-induced respiration increase [1] was fully inhibited by (R)-bromoenol lactone ((R)-BEL, 10 – 50 μM), a selective inhibitor of phospholipase iPLA$_2$γ,

whereas the glucose-induced part of respiration was amenable to full inhibition by pentose phosphate pathway inhibitors 6-aminonicotinamide (6-AN, 10 mM) and oxythiamine (OTA, 3 mM), and by NADPH oxidase (NOX) inhibitor diphenyleneiodonium chloride (DPI, 125 nM). Detection of diminishing mitochondrial membrane potential ($\Delta\Psi_m$) by tetramethylrhodamine ethyl ester (TMRE) assay paralleled respiratory experiments indicating that UCP2-mediated uncoupling stands behind the observed phenomena. These results are consistent with the presence of $iPLA_2\gamma$ in INS-1E cells and support the hypothesis that the activation of mitochondrial phospholipases by mild oxidative stress can provide free fatty acids as cycling substrates for UCP2. Supported by GACR grants No. P303/11/P320 (to J.J.) and P304/10/P204 (to A.D.).

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5P6

UCP3 in skeletal muscle mitochondria of hibernating ground squirrels does not transport pyruvate in contrast to the feature of UCP1 from brown adipose tissue

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Physiological role of UCP1 homologues still remains unclear. Among the numerous suggestions concerning possible functions of UCPs, hypothesis that UCP2 and UCP3, as carriers are involved in the coupling between glucose oxidation and mitochondrial metabolism has been recently proposed [1]. Export of pyruvate mediated by UCP2 and UCP3 could allow the cell to spare glucose and utilize fatty acids as the major mitochondrial energetic substrate.

In the present study we tried to investigate this hypothesis in the case of UCP3. It is known that expression of UCP3 in the skeletal muscles increases during the cold acclimatization, hibernation and starvation and correlates with increased fatty acid oxidation. We previously found increased UCP3 mRNA expression in skeletal muscle of hibernating squirrels compared to skeletal muscle of active squirrels [2]. We proposed that we would detect transport of pyruvate through UCP3 in the intact mitochondria of skeletal muscle of ground squirrels (*Spermophilus undulatus*) during the hibernation period.

Mitochondria of brown fat, where transport of pyruvate through UCP1 was clearly registered, were taken as a control. Valinomycin-induced swelling of non-respiring mitochondria in 55 mM potassium pyruvate was inhibited with 1 mM GDP. The presence of 1 mM α-cyano-4-hydroxycinnamate (α-CHC) eliminated the pyruvate carrier - mediated flux. In contrast, mitochondria of skeletal muscles were not able to demonstrate pyruvate transport in the similar conditions. At the same time, these mitochondria provided the functioning of pyruvate carrier, what was detected by nigericin-induced passive swelling in potassium pyruvate that was inhibited by α-CHC, and phosphate carrier, registered as nigericin-induced passive swelling in potassium phosphate.

Thus we could not reveal pyruvate transport through UCP3 in intact mitochondria of skeletal muscles of hibernating ground squirrels. Presented data as well as increasing literature data indicate the divergence in functional activity between UCP1 and its homologues. We believe further investigation of possible functions of UCP1 homologues are required.

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5P7

Absence of uncoupling protein-3 (UCP3) affects mice metabolic parameters and the metabolic adaptation induced by the administration of triiodothyronine to hypothyroid rats

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Triidothyronine (T3) influences metabolic rate, heat production and lipid metabolism, but the molecular determinants underlying its effects are still under investigation. When T3 was administered to hypothyroid rats a strict correlation in terms of time course between induced increases in UCP3 protein levels, mitochondrial uncoupling, and resting metabolic rate (RMR) has been observed; thus suggesting that UCP3 could be a molecular determinant for regulation of the RMR by T3. However, a debate exists about the function of UCP3, and new data should be obtained in order to ultimately characterize the involvement of UCP3 in the metabolic effects elicited by T3. The use of mice lacking of UCP3 can help to clarify this aspect.

To this aim we used wild-type (WT) and UCP3-null (KO) mice maintained at thermoneutrality (30 °C). We detected whole animals RMR, Heat Production (HP) and Respiratory Quotient (RQ), as well as proton conductance and fatty acid oxidation rate in isolated skeletal muscle mitochondria. In addition, to evaluate the possible role of UCP3 in T3-induced metabolic adaptations, we injected a single dose of T3 (25 microgr/100 g bw) into both WT and KO hypothyroid mice and we measured their RMR, HP and RQ.

KO mice showed significantly lower RMR (−20%) and HP (−23%), as well as an higher RQ (+5%) compared to WT, thus suggesting the involvement of UCP3 in mice metabolism and in the ability of the animal to use lipids as metabolic substrate. Results obtained in the whole animal are accordance with that obtained in isolated skeletal muscle mitochondria, as KO mice mitochondria showed inhibited fatty acid-induced proton-conductance and fatty acid oxidation rate compared to WT ones.

The administration of T3 into hypothyroid mice induced an enhancement in their RMR and HP as well as a reduction in RQ. However, differences between WT and KO mice were observed in terms of time course and degree of the T3-induced variations, that were more prolonged and higher in WT mice. Indeed, 48 h following the administration of T3 i) the increases in RMR were +17% and +10%, in WT and KO mice, respectively ii) the enhancement in HP were +20% and +7% in WT and KO mice, respectively and iii) a significant decrease in RQ (−6%) was present exclusively in WT mice.